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Comparison of broad-range bacterial PCR and culture of cerebrospinal fluid for diagnosis of community-acquired bacterial meningitis

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ABSTRACT

Appropriate, rapid and reliable laboratory tests are essential for the diagnosis and optimal antibiotic therapy of acute bacterial meningitis. Broad-range bacterial PCR, combined with DNA sequencing, was compared with culture-based methods for examining cerebrospinal fluid (CSF) samples from patients with suspected meningitis. In total, 345 CSF specimens from 345 patients were analysed, with acute community-aquired bacterial meningitis being diagnosed in 74 patients. The CSF of 25 patients was positive by both PCR and culture; 26 patients had CSF specimens positive by PCR only, and 14 patients had specimens positive by culture only. The sensitivity of PCR and culture for clinically relevant meningitis was 59% (44 / 74) and 43% (32 / 74), respectively, while the specificity was 97% (264 / 271) and 97% (264 / 271), respectively. The commonest bacterial rRNA gene sequences detected by PCR only were those of *Streptococcus pneumoniae* and *Neisseria meningitidis* ($n = 12$). PCR failed to detect the bacterial rRNA gene in seven specimens from patients with symptoms compatible with acute bacterial meningitis. Overall, the results demonstrated that PCR in conjunction with sequencing may be a useful tool in the diagnosis of bacterial meningitis. PCR is particularly useful for analysing CSF from patients who have been treated with antibiotics before lumbar puncture.

Keywords Broad-range PCR, diagnosis, meningitis, PCR, rRNA gene, screening

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INTRODUCTION

Acute bacterial meningitis is a rapidly progressing serious disease with high morbidity and mortality if treated incorrectly. Meningitis is usually suspected on the basis of the clinical presentation of the patient and the finding of pleocytosis with dominance of polymorphonuclear cells in the cerebrospinal fluid (CSF) at lumbar puncture. Adequate treatment requires rapid detection and identification of the bacteria, as permanent neurological sequelae (e.g., hearing loss, mental retardation, seizures and behavioural changes) may occur in up to 50% of survivors. Current diagnostic methods, i.e., direct microscopy and culture, may be insufficient in certain circumstances, and results from culture are commonly not available for at least 24–48 h. Some patients receive antibiotics before lumbar puncture, resulting in negative CSF cultures, and meningitis may also be caused by fastidious or slow-growing microorganisms that are difficult to detect by culture. Rapid diagnosis of the aetiological agent is also particularly important, for epidemiological reasons, in cases of meningococcal disease, where secondary cases are not uncommon.

During the last two decades, PCR-based assays for providing an early and accurate diagnosis of bacterial meningitis have become available [1–7]. Broad-range bacterial PCR is

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based on the use of primers that recognise conserved regions of the gene coding for 16S rRNA. The resulting amplified rDNA also contains species-specific variable regions that provide a reliable basis for estimating phylogenetic relationships among bacteria [8]. Detection of the 16S rRNA gene can be used to indicate the presence of bacteria in specimens from usually sterile locations, with the precise bacterial species being identified by subsequent sequencing. The present study describes a PCR strategy for the general detection of bacteria in CSF specimens, followed by cycle sequencing to identify the bacterial species involved.

PATIENTS AND METHODS

Patients

All CSF samples sent for culture to the Bacteriological Laboratory, Sahlgrenska University Hospital, Göteborg, Sweden and two adjacent local laboratories (NU-sjukvården, Uddevalla and SÄS, Borås) in the same county during a 4-year period (1999–2002) were considered eligible for the study if the total CSF white blood cell (WBC) count was $\geq 10 \times 10^6$ / L, indicating meningeal inflammation. The data concerning CSF WBC counts were obtained directly from the Department of Clinical Chemistry. Only the first CSF sample from each patient was included. Acute bacterial meningitis was defined as meningitis caused by either *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Listeria* spp., Gram-negative bacilli, streptococci or staphylococci, as verified by culture from CSF or blood, and / or clinical signs, e.g., headache, fever and meningismus, compatible with bacterial meningitis. Samples from patients with nosocomial central nervous system (CNS) infections or inflammations, defined as an onset of symptoms ≥3 days following hospitalisation (i.e., shunt infections and post-operative CNS infections), were not included in this study.

Medical records of all patients included in the study were reviewed retrospectively for a final diagnosis, predisposing factors, treatment and outcome by one of the authors (LD). Final diagnoses were: acute community-acquired bacterial meningitis (n = 74); aseptic meningitis of suspected viral origin (n = 102); encephalitis (n = 22); Lyme neuroborreliosis with meningitis (n = 78); *Mycoplasma* meningoencephalitis (n = 7); mycobacterial meningitis (n = 4); helmininthic meningitis (n = 1); cryptococcal meningitis (n = 1); parainfectious meningeal inflammation (CNS abscesses) (n = 14); non-infectious meningeal inflammatory reaction (n = 37); and malignancy with meningeal involvement (n = 5). The relevance of the PCR findings and bacterial cultures to the final diagnosis was evaluated and compared with the clinical findings and other laboratory results.

The study included 345 CSF samples from 345 patients (51% female) with a CSF WBC count $\geq 10 \times 10^6$ / L. The median age of the patients was 34 years (range 1 day-91 years), and 72 patients had severe underlying conditions, of which the most common were pre-term delivery (n = 24), malignant disease (n = 16), diabetes mellitus (n = 13), alcohol abuse (n = 8) and renal insufficiency (n = 3).

Preparation of specimens for PCR

All PCR analyses were performed at the Göteborg laboratory. CSF samples (50 μL - 1.5 mL) were centrifuged at 12 000 g for 20 min, after which DNA was extracted from the pellet with a bacterial DNA preparation kit (Roche, Stockholm, Sweden), used according to the manufacturer's instructions.

DNA amplification techniques

The primers used to amplify a segment of the 16S rRNA gene were as described previously. Forward primer SSU1 [9], 5′-CGGCAGGCCTAACACATGCAAGTCG, and reverse primer 806R [10], 5′-GGACTACCAGGGTATCTAAT, are complementary to conserved regions in the 5′ region of the 16S rRNA gene, producing a fragment of *c*. 766 bp (nucleotide positions 41–806 in *Escherichia coli* [11]).

PCR mixtures contained 50 mM Tris-HCl, pH 8.0, bovine serum albumin 0.5 mg / mL, 4 mM $MgCl_2$, 100 mM each dNTP, 2 U of *Taq* DNA polymerase (Applied Biosystems, Stockholm, Sweden) and 0.5 μM each primer (CyberGene, Huddinge, Sweden). Reaction mixtures were illuminated with UV light at 312 nm (8 W) for 10 min in the reaction tubes (40-μL aliquots) to eradicate any DNA contaminating the mastermix, after which 10 μL of DNA extracted from the clinical specimen was added. Amplification was performed using a DNA Thermal Cycler 2400 (Applied Biosystems), with 40 cycles of 15 s at 94°C, 30 s at 55°C and 1 min at 72°C, followed by 10 min at 72°C. Aliquots (15 μL) of PCR products were separated in agarose 2% w / v gels stained with ethidium bromide, and compared with the amplicon generated from *Staphylococcus aureus*. DNA bands of the expected size (766 bp) were cut from the gel, purified with a Qiaquick Gel Extraction kit (Qiagen, Göteborg, Sweden) and subjected to cycle sequencing using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit, v.1.1 (Applied Biosystems). The sequencing reaction products were analysed using an ABI PRISM 310 Genetic Analyser (Applied Biosystems).

After DNA sequence editing, the GenBank BLAST program was used for sequence comparisons [12]. The sequences obtained from patient specimens matched sequences in the database, producing significant alignments and providing a BLAST result, score bits and *E*-value, indicating the probability that the match observed was not consistent with chance. Weak PCR signals giving BLAST similarity results of <97% were considered to be negative.

Analytical sensitivity of PCR

Strep. pneumoniae, *Staph. aureus* and *E. coli* cultures were diluted ten-fold in sterile phosphate-buffered saline and viable counts were determined. The bacteria were then added to CSF (culture- and PCR-negative) to determine the analytical sensitivity of the assay.

In total, 105 clinical isolates of a wide variety of Gram-positive and Gram-negative bacteria isolated from CSF or blood cultures in the Göteborg laboratory were used to confirm that the primers detected bacteria associated with

bacterial meningitis or sepsis. The primers also amplified DNA from *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*. These isolates are available from the Culture Collection of the University of Göteborg (http://www.ccug.se).

Culture

CSF samples were submitted for routine bacterial culture and chemistry [5]. Other diagnostic procedures, depending on the clinical circumstances, included CSF cultures for mycobacteria and fungi, CSF cytology and protein electrophoresis, blood cultures, serological tests for *Mycoplasma*, *Borrelia* and *Toxoplasma*, and serological tests and / or PCRs specific for neurotropic viruses, e.g., herpes simplex virus, varicella-zoster virus and tick-borne encephalitis virus, according to the clinicians' requests. These additional diagnostic procedures were not performed systematically for all samples, and only culture results for rapidly growing aerobic and anaerobic bacteria were included in the comparison with PCR and sequencing. The sensitivity of PCR vs. bacterial culture was compared using McNemar's test. For the subgroup treated with antibiotics, Fisher's exact test was used.

RESULTS

PCR-positive samples

The analytical sensitivity of the PCR assay was 10 CFU / mL for *Strep. pneumoniae*, and 1×10^3 CFU / mL for *Staph. aureus* and *E. coli*. Of 345 clinical specimens, 25 (7%) were positive by both PCR and culture (Table 1). The PCR and culture results for the CSF specimens were confirmed by culture of blood or samples from other sterile locations for 17 of these 25 patients.

Bacterial DNA was amplified and sequenced from culture-negative CSF samples from 26 patients (Table 2). In six cases, DNA from *Strep. pneumoniae* was identified, with the pneumococcal aetiology being supported by the isolation of this organism from blood in five of these six cases. DNA from *N. meningitidis* was identified in CSF from six patients, with growth of this organism from blood of one of the patients. Of the remaining 14 PCR-positive, culture-negative samples, the blood culture and CSF PCR results were concordant in four cases. DNA from *Streptococcus agalactiae* was identified in one sample. Two samples that were PCR-positive for *Staphylococcus haemolyticus* and *Staphylococcus epidermidis*, respectively, probably represented contaminants, since other well-known pathogens were isolated from blood cultures of these patients. PCR results for another five patients were also considered to represent contamination, as two patients had clinical and serological indications of Lyme neuroborreliosis, one had *M. pneumoniae* encephalitis, and two had viral encephalitis. Finally, DNA from *Fusobacterium* and *Granulicatella*, respectively, was amplified from two patients with clinical and laboratory signs of bacterial meningitis, with very high CSF

Table 1. Bacteria detected by both PCR and culture in cerebrospinal fluid (CSF) from 25 patients with acute bacterial meningitis

Bacteria in CSF	Same result from culture of blood or other sterile site	Patient age/gender	CSF leukocytes, poly/mono ($\times 10^6$ / L)	Antibiotic treatment before sampling
Streptococcus pneumoniae	Yes	55 years / F	90 / 113	No
Streptococcus pneumoniae	Yes	43 years / F	146 / 16	No
Streptococcus pneumoniae	Yes	64 years / M	6400 / NA	No
Streptococcus pneumoniae	Yes	58 years / F	81 / 32	No
Streptococcus pneumoniae	Yes	3 years / M	409 / 97	No
Streptococcus pneumoniae	Yes	51 years / F	142 / 174	Cefotaxime IV
Streptococcus pneumoniae	Not done	56 years / F	3225 / 410	No
Streptococcus pneumoniae	Yes	56 years / M	16600 / 3700	Fenoximethylpenicillin PO
Streptococcus pneumoniae	Yes	41 years / F	3480 / 240	No
Streptococcus pneumoniae	Not done	85 years / M	5350 / 150	No
Neisseria meningitidis	Negative	16 years / F	2295 / 156	No
Neisseria meningitidis	Yes	5 months / F	900 / 676	No
Neisseria meningitidis	Yes	7 months / F	NA / 3000	No
Neisseria meningitidis	Yes	15 years / F	117 / 40	Cefotaxime IV
Neisseria meningitidis	Not done	10 years / M	4380 / NA	No
Neisseria meningitidis	Yes	14 years / F	11200 / 1500	No
Haemophilus influenzae	Yes	76 years / F	2720 / 100	No
Haemophilus influenzae	Yes	1 month / F	1700 / 620	No
Haemophilus influenzae	No	29 years / M	5700 / 2500	Cefadroxil PO
Listeria monocytogenes	No	59 years / M	2074 / 209	Ampicillin IV
Listeria monocytogenes	Not done	59 years / F	810 / 125	No
Staphylococcus aureus	Yes	84 years / M	1280 / 340	Cloxacillin IV, fusidic acid PO, cefuroxime IV
Staphylococcus aureus	Yes	43 years / M	630 / 90	No data
Streptococcus pyogenes	Yes	36 years / M	304 / 122	No
Citrobacter koseri	Not done	2.5 months / M	2500 / 700	Cefotaxime IV

M, male; F, female; NA, not available; IV, intravenous; PO, oral.

Table 2. Bacteria detected by PCR only in cerebrospinal fluid (CSF) from 26 patients

Bacterial DNA in CSF	Same result from culture of blood or other sterile site	Patient age/gender	CSF leukocytes poly/mono ($\times 10^6$/L)	Antibiotic treatment before sampling	Final diagnosis
Clinically relevant finding					
Streptococcus pneumoniae	Negative	42 years / F	3160 / 800	Amoxyclav PO	BM
Streptococcus pneumoniae	Yes	50 years / F	Not available	No	BM
Streptococcus pneumoniae	Yes	78 years / M	3330 / Not available	Cefotaxime IV	BM
Streptococcus pneumoniae	Yes	91 years / M	3540 / 760	Cefuroxime IV	BM
Streptococcus pneumoniae	Yes	47 years / F	786 / 243	Cefotaxime IV	BM
Streptococcus pneumoniae	Yes	61 years / F	2 / 6	Cefuroxime IV	BM
Neisseria meningitidis	Yes	36 years / M	3560 / 1780	No	BM
Neisseria meningitidis	Negative	6 months / F	9 / 23	No	BM
Neisseria meningitidis	Negative	57 years / M	840 / 2260	No	BM
Neisseria meningitidis	Negative	23 years / M	9000 / 900	Cefotaxime IV	BM
Neisseria meningitidis	Not available	29 years / F	7300 / 600	Cefotaxime IV	BM
Neisseria meningitidis	No	15 years / F	5510 / 1250	Cefuroxime IV	BM
Escherichia coli	Yes	73 years / F	1719 / 620	Cefuroxime IV	BM
Fusobacterium necrophorum	No	68 years / M	5500 / 2300	Cefuroxime IV	BM
Granulicatella adiacens	No	61 years / F	800 / 3800	Cefotaxime + ampicillin IV	BM
Klebsiella pneumoniae	Yes	35 years / M	2310 / 360	Amoxyclav PO	BM
Streptococcus agalactiae	Negative	87 years / F	1680 / 160	Cefuroxime IV	BM
Streptococcus anginosus	Yes	65 years / M	124 / 118	Cefuroxime IV	Endocarditis septical emboli to CNS
Streptococcus intermedius	Yes	26 years / M	1540 / 200	Cefuroxime IV	Spondylitis, abscess of the psoas muscle, secondary BM
Finding not clinically relevant					
Acinetobacter spp.	Not done	8 years / M	151 / 59	No	Viral meningitis
Actinomyces spp.	Not done	5 years / F	1 / 37	No	Neuroborreliosis
Enterococcus spp.	Not done	7 years / F	1 / 20	No	Neuroborreliosis
Staphylococcus aureus	No	10 years / M	56 / 45	Cefotaxime IV	*Mycoplasma* encephalitis
Staphylococcus haemolyticus	*Cryptococcus*	59 years / F	219 / 423	No	Cryptococcal meningitis
Staphylococcus epidermidis / hominis	*Salmonella*	27 years / M	8 / 19	Cefuroxime IV	HIV-positive toxoplasmosis
Streptococcus pyogenes	No	38 years / M	0 / 20	No	Encephalitis of unknown origin

M, male; F, Female; IV, intravenous; PO, oral; BM, bacterial meningitis; HIV, human immunodeficiency virus; CNS, central nervous system.

WBC counts, but there were no other findings supporting the significance of these DNA amplification results.

PCR-negative samples

Bacteria were detected by culture from 14 CSF specimens, but no bacterial DNA was detected by PCR (Table 3). The culture results were considered to be clinically relevant in seven cases, as well-known pathogens were isolated (*Staph. aureus*, *Listeria monocytogenes* and *Strep. pneumoniae*) and no other causes of CNS infection were demonstrated. In the remaining seven patients, the organisms isolated from CSF were probably contaminants (six isolates of coagulase-negative staphylococci and one of *Ralstonia pickettii*) and other causes of CNS infection were found (Table 3).

Bacteria were identified neither by PCR nor culture in 280 CSF specimens. In seven cases, both methods probably failed to detect an aetiological agent of bacterial meningitis, as these patients had clinical and laboratory findings suggestive of bacterial meningitis, and a relevant pathogen was isolated from blood culture (*Staph. aureus*, $n = 4$; *E. coli*, $n = 2$; *H. influenzae*, $n = 1$).

Seventy-eight patients with a negative PCR result had *Borrelia* meningitis, verified by demonstration of *Borrelia* antibodies in CSF or by significant increases in *Borrelia* antibodies in paired sera. Thus, 78 (23%) of 345 patients had Lyme neuroborreliosis, 75 with a negative PCR and culture, one with a positive culture considered to be a contaminant, and two with positive PCR results caused by contaminating bacteria (Tables 1 and 2). Four patients with negative PCR and culture results had *M. pneumoniae* meningoencephalitis, verified by detection of specific IgM antibodies in serum. As mentioned above (Tables 1 and 2), another three patients had *Mycoplasma* meningoencephalitis, with signs of contaminating bacteria according to culture or PCR. Three patients had CSF specimens positive for *Mycobacterium tuberculosis*, and one patient was positive for atypical mycobacteria.

Viral CNS infection was suspected or verified in 124 patients; 102 of these patients had viral meningitis and 22 had signs of encephalitis

Table 3. Bacteria detected only by culture in cerebrospinal fluid (CSF) from 14 patients

Bacteria in CSF	Same result from culture of blood or other sterile site	Patient age/gender	CSF leukocyte poly/mono ($\times 10^6$/L)	Antibiotic treatment before sampling	Final diagnosis
Clinically relevant finding					
Staphylococcus aureus	Yes	63 years / M	60 / Not available	Cefuroxime IV	Septicaemia and BM
Staphylococcus aureus after enrichment	Not done	88 years / F	49 / 12	Cefuroxime IV	Probably septicaemia, meningeal irritation
Staphylococcus aureus	Yes	61 years / M	420 / 140	Cefotaxime IV	BM
Staphylococcus aureus after enrichment	No	86 years / M	1100 / 660	Fenoximethyl-penicillin PO	BM
Listeria monocytogenes	No	84 years / M	646 / 203	No	BM
Listeria monocytogenes	Not done	28 years / F	1500 / 170	No	BM
Streptococcus pneumoniae after enrichment	No	47 years / F	154 / 678	Fenoximethyl-penicillin PO	BM
Finding not clinically relevant					
CoNS	No	17 years / F	234 / 75	Doxycycline PO	*Mycoplasma* encephalitis
CoNS	Not available	41 years / F	6 / 179	No	Herpes simplex encephalitis
CoNS	No	71 years / M	0 / 14	No	Cerebral lymphoma
CoNS	No	38 years / F	0 / 20	No	Multiple sclerosis, no infection
CoNS	No	31 years / M	1293 / 40	No	BM, probably not CoNS
CoNS	No	32 years / M	2 / 202	No	*Borrelia*
Ralstonia pickettii	No	22 years / M	93 / 97	Not available	*Mycoplasma encephalitis*

M, male; F, female; IV, intravenous; PO, oral; BM, bacterial meningitis; CoNS, coagulase-negative staphylococci.

(including seven with tick-borne encephalitis and five with herpes simplex type 1 encephalitis). One patient had an eosinophilic meningitis caused by the helminth *Gnathostoma spinigerum*. For 56 patients, other reasons existed for high CSF cell counts, e.g., aseptic systemic inflammatory diseases, parainfectious meningeal reactions (caused by brain abscesses), malignancies or sudden infant death syndrome.

Treatment with antibiotics

Of the 25 patients for whom CSF specimens were positive by both PCR and culture (Table 1), seven had received empirical antibiotic therapy before lumbar puncture, i.e., two patients with *Strep. pneumoniae*, one with *Staph. aureus*, one with *N. meningitidis*, one with *L. monocytogenes*, and one with *Citrobacter koseri*. The seventh patient, infected by *H. influenzae*, was given empirical treatment with cefadroxil. No information on the possible use of antibiotics before lumbar puncture was available for one patient. In contrast, 15 (79%) of 19 culture-negative CSF specimens with clinically relevant PCR-positive results (Table 2) were from patients who had received empirical antibiotic therapy before lumbar puncture, i.e., five of six patients with *Strep. pneumoniae*, three of six patients with *N. meningitidis* and seven patients with either *Granulicatella adiacens*, *Klebsiella pneumoniae*, *Strep. agalactiae*, *Streptococcus anginosus* or *Streptococcus intermedius*.

Table 4. Results of PCR with sequencing and culture in comparison with clinical diagnosis of acute bacterial meningitis

	Acute bacterial meningitis	No acute bacterial meningitis[a]	Total
PCR[b]			
Positive	44	7	51
Negative	30	264	294
Total	74	271	345
Culture[c]			
Positive	32	7	39
Negative	42	264	306
Total	74	271	345

[a]Includes 78 patients with a diagnosis of neuroborreliosis and four patients with mycobacterial infection.
[b]PCR: sensitivity 59% (44 / 74); specificity 97% (264 / 271); positive predictive value 86% (44 / 51); negative predictive value 91% (264 / 294).
[c]Culture: sensitivity 43% (32 / 74); specificity 97% (264 / 271); positive predictive value 82% (32 / 39); negative predictive value 86% (264 / 306).

Sensitivity and specificity

The sensitivity of PCR for detecting bacterial meningitis was higher than that of culture (59% (44 of 74) vs. 43% (32 of 74); $p < 0.05$, McNemar's test) (Table 4). The specificity of PCR was high (97%; 264 of 271), and was as high as that of culture, even though virtually all patients with some kind of meningeal inflammation were included in the evaluation. If both culture and PCR were used for detection of bacterial meningitis, the sensitivity was 69% (51 of 74). The difference in the sensitivities between PCR (79%) and culture (45%) among patients treated with antibiotics was also statistically significant ($p < 0.005$, Fisher's exact test).

DISCUSSION

The present study illustrates both the strengths and the limitations of broad-range bacterial PCR for detection of aetiological agents involved in bacterial meningitis. PCR is rapid and able to detect low numbers of bacteria in CSF, and non-viable aetiological agents can be detected despite the fact that empirical antimicrobial therapy may have been administered before lumbar puncture. However, like culture, PCR may also detect contaminating bacteria and give false-positive results. DNA from certain bacteria (e.g., Gram-positive cocci) in CSF seems to be more difficult to extract and requires additional preparation. False-negative results may be obtained if more than one aetiological bacterial species is present. This may occur particularly in cases of post-operative or shunt-related CNS infections.

PCR has been a helpful diagnostic tool in clinical microbiology for more than a decade [13–15]. With the successful development of new techniques and instruments, sequencing has become a relatively quick procedure. However, the impact of systematic sequencing of non-cultivable bacteria in a clinical laboratory setting still needs to be evaluated critically. The results of PCR and sequencing can be available within 30 h of the arrival of a specimen in the laboratory. Isolation of bacteria by culture may require 24–48 h, and may sometimes be hampered by empirical antibiotic therapy before lumbar puncture.

In the present study, 345 CSF specimens from 345 consecutive patients with signs and symptoms of acute meningitis were analysed by culture and broad-range PCR combined with sequencing in order to determine the clinical utility of such techniques for rapid diagnosis and as a guide to the clinician in making decisions concerning antimicrobial therapy. The molecular methods used in this study are faster and more discriminatory than, e.g., hybridisation techniques or techniques using semi-nested PCR or restriction enzyme digestion instead of sequencing [2,3,16]. Probe-based real-time PCR is the method of choice when using a multiplex PCR for simultaneous detection of different specific target sequences. However, real-time PCR does not appear to offer advantages when performing a broad-range PCR [7,17,18]; indeed, there may be disadvantages, including possible cross-reactions with human DNA and a need for DNA clean-up before sequencing to avoid any fluorescent interference.

The present study used conventional PCR followed by agarose gel electrophoresis in order to visualise and selectively purify specific amplicons. In this way, irrelevant PCR products, e.g., primer dimers or products originating from human DNA, could be avoided and good-quality DNA could be obtained for sequencing. Xu *et al*. [5] showed that the combination of broad-range conventional PCR and sequencing was comparable to specific real-time PCR for the detection of *N. meningitidis*. Real-time PCR using specific primers may complement broad-range PCR for detection of the most common pathogens causing bacterial meningitis, which would circumvent otherwise necessary sequencing. However, culture will continue to be required to facilitate susceptibility testing.

In addition to detecting the 25 bacterial species identified by culture, PCR and sequencing detected another 19 culture-negative cases of acute bacterial meningitis. Of these, 15 patients had received antibiotics before lumbar puncture, confirming that PCR amplification may detect non-viable bacteria. This may also be the reason why several cases of bacterial meningitis caused by *Strep. pneumoniae* were detected only by PCR, as pneumococci have a tendency to autolyse in culture media. In contrast, of the 25 patients with CSF specimens positive by both PCR and culture, only five had been given antibiotics before lumbar puncture.

Gran. adiacens and *Fusobacterium necrophorum* were identified only by PCR and sequencing in CSF samples from two patients. These two organisms are known to be fastidious and may be difficult to detect by culture. These two patients showed symptoms of acute bacterial meningitis; no other bacteria were detected, and no other underlying diseases were present. There are several reports of *Fusobacterium* as a cause of bacterial meningitis [19,20], but reports describing *Granulicatella* as a causative agent are rare [21], perhaps because detection systems have not been sufficiently sensitive. It is not always possible to state with certainty whether an organism of low virulence is a relevant pathogen or a contaminant. The detection of DNA from *Gran. adiacens* and other streptococci-like bacteria in the present study should therefore be regarded with caution.

The PCR technique failed to identify seven cases of bacterial meningitis; in two of these cases, *L. monocytogenes* was identified by culture. It is known that *Listeria* produces symptoms of bacterial meningitis even when present only in low (40–400 CFU / mL) numbers in CSF [22]. This could explain why PCR failed to identify the bacteria in these specimens, as also reported in two other studies [6,23]. A nested or semi-nested PCR might improve the sensitivity by up to 100-fold, which would help to solve this problem [23,24], but the laboratory would need to be informed in advance by the clinician that *Listeria* infection was suspected.

During the present study it became apparent that the method for preparing DNA from a diversity of bacterial species, many of which are encapsulated, required further improvement. All specimens prepared for the detection of bacterial DNA of unknown bacterial species are now treated with mutanolysin and lysostaphin to effectively destroy the cell wall [25,26]. The failure to detect *Staph. aureus* by PCR in four CSF specimens and *Strep. pneumoniae* in one (Table 3) might be explained by inadequate extraction of DNA. One specimen containing *Strep. pneumoniae* and two of four specimens containing *Staph. aureus* were detected only by culture after cultivation in enrichment broth, which probably reflected low numbers of bacteria in the CSF. In general, 2 mL of the CSF specimen was used for culture, and the remainder (often <500 μL) was used for PCR, which might explain the decreased sensitivity of the PCR. It is therefore recommended that at least 1 mL of CSF is used for PCR analysis, even though *N. meningitidis* was detected in a volume of *c.* 50 μL. The importance of the volume of CSF analysed has not been addressed adequately in previous studies, which have usually focused on detection of *H. influenzae*, *N. meningitidis* and *Strep. pneumoniae*, all of which are often present in high numbers in the CSF of patients with meningitis [3,6].

PCR and culture gave concordant negative results for 280 specimens, of which 261 were from patients whose final diagnosis was not acute bacterial meningitis, with high WBC counts being caused by virus infections or other factors. Seventy-eight of these patients had tick-borne *Borrelia burgdorferi sensu lato* meningitis. Detection of *Borrelia* spirochaetes in CSF is not possible by PCR targeted at the 16S rRNA gene of *Borrelia* spp. Seven patients had *Mycoplasma* meningitis according to serological findings, although no bacteria were identified in CSF by PCR. Sensitivity is, to some extent, dependent on the number of copies of the 16S rRNA gene. As *M. pneumoniae* has only one gene coding for 16S rRNA [27], this organism is more difficult to detect than (e.g.) *E. coli*, which has seven copies of the 16S rRNA gene [28]. An additional eight CSF specimens gave very weak PCR results, and the subsequent low-quality DNA sequences resulted in GenBank similarities of <97%. These were considered to be negative results, as this percentage is the lowest acceptable similarity for a bacterial isolate to be identified to a known species [29]. Such identifications cannot be reported to the clinician as a reliable result.

It has been suggested previously that PCR can be used for diagnostic purposes when patients have been treated with antibiotics, since both dead and viable microorganisms can be detected [5,6], and this was confirmed in the present study. The broad-range PCR described in this study has been tested with CSF specimens in a clinical setting for a 3-year period. Following successful comparisons with laboratory findings and clinical symptoms, the PCR method is now used in clinical practice to complement direct microscopy and culture, especially when patients with suspected bacterial meningitis have been treated with antibiotics before sampling.

In summary, the present study demonstrated that broad-range PCR combined with sequencing may be valuable for diagnosing and identifying the aetiology of acute community-acquired bacterial meningitis. The method is especially useful with CSF specimens from patients who have been given empirical antibiotics before the lumbar puncture. The technique is relatively fast and may be used routinely. Using ≥1 mL of CSF, the sensitivity is adequate for the detection of most bacteria. However, *Borrelia*, *Listeria* and *Mycoplasma* may be present in CSF in low numbers, and may have few copies of the 16S rRNA gene; therefore, if these bacteria are suspected, a nested PCR approach is preferable.

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